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Effects of Additives on the Stability of *Humicola lanuginosa* Lipase during Freeze-Drying and Storage in the Dried Solid

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Abstract □ The effects of various classes of additives on the stability of a protein with a relatively hydrophobic surface, *Humicola lanuginosa* lipase (HLL), during lyophilization and storage in the dried solid, were investigated. Prior to lyophilization, it was found that 1 M trehalose or 1% (wt/vol) Tween 20 caused the protein to precipitate. Infrared spectroscopy indicated that trehalose "salted-out" native HLL, whereas Tween 20 induced non-native aggregates. Optimal recovery of native protein in the initial dried solid was obtained in the presence of additives which formed an amorphous phase and which had the capacity to hydrogen bond to the dried protein (e.g., trehalose and sucrose). Additives which crystallized during lyophilization (e.g., mannitol) or which remained amorphous, but were unable to hydrogen bond to the dried protein (e.g., dextran), afforded less stabilization relative to that seen in the absence of additives. Optimal storage stability in the dried solid required that both protein unfolding during lyophilization was minimized and that the formulation was stored at a temperature below its T_g value. Crystallization of sucrose during storage greatly reduced the storage stability of HLL. This was attributed to the increased moisture content and the reduced T_g value in the remaining amorphous phase containing the protein. Sucrose crystallization and the resulting damage to the protein were inhibited by decreasing the mass ratio of sucrose:protein.

Introduction

Mechanisms that are proposed to account for stabilization of proteins acutely during lyophilization and long-term during storage in the dried solid are based mainly upon studies using globular proteins with a typical hydrophilic surface. It has been found that, in addition to formation of an amorphous phase in the dried solid, the ability of the additive to hydrogen bond to the dried protein and thus act as a water substitute also is required for optimal protection of a protein during the lyophilization process.[1] Furthermore, it has been found that long-term storage stability depends on two factors: (1) formation of a an amorphous phase containing protein and additive and maintenance of the formulation glass transition temperature (T_g) above the storage temperature, and (2) preservation of native, aqueous protein secondary structure during lyophilization.[2-4]

To determine if the same mechanisms may account for stabilization of a soluble, globular protein which has a much more hydrophobic surface than proteins typically studied, the current study investigates the effects of additives with selected properties on the acute and long-term stability of lyophilized *Humicola lanuginosa* lipase (HLL). HLL is a well-characterized protein[5,6] that has a predominantly hydrophobic surface exposed to the solvent, as determined by hydrophobicity plots.[7] HLL is used as an

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enzymatic detergent additive[8] and has shown potential as a therapeutic protein for exocrine pancreatic insufficiencies.[9] The additives tested were mannitol, dextran, trehalose, and sucrose.

Sugars are often used to stabilize proteins during lyophilization and during subsequent storage in the dried solid. However, similar to other reports,[10-12] we have recently observed that crystallization of a sugar from the amorphous state during storage can drastically reduce protein storage stability.[2] Crystallization of amorphous sugars is usually only observed on a reasonable time scale of weeks to months during storage at temperatures close to or above the formulation T_g value.[13] Crystallization can be due to increased moisture contents (e.g., because of moisture transfer from the vial stopper to the dried formulation)[14,15] or simply due to prolonged storage.[16] Recently, it has been shown that proteins, at an adequately high protein:sugar mass ratio, are able to prevent crystallization of either sucrose[17] or trehalose[18] from the dried solid. However, since the T_g value of sucrose is much lower than that of trehalose at similar moisture contents,[19,20] it is expected that sucrose may crystallize at lower temperatures than trehalose. Thus, another objective of the current study is to determine the effects of varying protein:sugar mass ratios on the capacity of HLL to inhibit crystallization of sucrose or trehalose during storage.

In addition to issues involving sugar crystallization, it is generally of interest to investigate the relative stabilizing effects of sucrose or trehalose on the acute and long-term stability of proteins. This topic has been the subject of many publications.[12,19-21] Trehalose has been shown to provide improved storage stability at high storage temperatures to proteins[2,22] and to liposomes[20] compared to that provided by sucrose. However, no study to date has addressed the relative effectiveness of the disaccharides as stabilizers for a protein with a relatively hydrophobic surface, such as HLL.

Materials and Methods

Materials—Recombinant *Humicola lanuginosa* lipase (HLL), expressed in *Aspergillus oryzae*,[5] was a kind gift from Novo Nordisk A/S (Bagsværd, Denmark). The protein was obtained as a lyophilized, additive-free powder containing >95% HLL (by activity assay). Potassium phosphate, mannitol, dextran (*Leuconostoc mesenteroides*, Strain no. B-512, Average MW 162 kDa) and poly(oxyethylene) sorbitan monolaurate (Tween 20) were obtained from Sigma Chemical Co. (St. Louis, MO). Sucrose and trehalose were purchased from Pfanstiehl Laboratories (Waukegan, IL). All other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO), except as indicated below.

Formulation Preparation—HLL was dissolved in 10 mM potassium phosphate buffer (pH 10, at 23 °C) at 4 mg/mL ($E_{280\ nm}$ (1%) = 14.68)[6] and mixed in a 1:1 (vol:vol) ratio with the respective additive solutions (prepared at twice the desired concentration using phosphate buffer) to obtain a final HLL concentration of 2 mg/mL and the final desired additive concentration. Formulations were subsequently aliquotted (1.0 mL) into 5 mL lyophilization vials (21 mm ID) (West Company, Lionsville, PA).

Lyophilization—The protein formulations were lyophilized in a FTS Durastop μP microprocessor controlled freeze-drier (Stoneridge, NY) as previously described.[2] After drying was complete, the vials were capped under vacuum using butyl stoppers from the West Company (formulation no. 4416) (Lionsville, PA). None of the formulations showed any evidence of cake collapse following lyophilization.

Storage Studies—Following lyophilization, vials were incubated at 40 or 60 °C and analyzed following 0, 14 days, 1 and 3 months. Control formulations were stored at −80 °C and analyzed following 3 months. Following storage, formulations were rehydrated to 1 mg/mL HLL with deionized, distilled water and filtered to remove insoluble aggregates, and the filtrate was analyzed as described below. Triplicate vials from each formulation were assayed at each time point. No differences were seen between formulations assayed at $t = 0$ and the control formulations stored at −80 °C (results not shown).

Analytical Methods—*Size exclusion high performance liquid chromatography (HPLC-SEC)* was performed by using a Dionex HPLC (Sunnyvale, CA) equipped with a GP40 gradient pump and an AD20 detector, using a detection wavelength of 215 nm. The amount of native, monomeric protein molecules and non-native soluble aggregates were quantitated using a Zorbax G250 column (MACMOD Analytical Inc., Chadds Ford, PA) eluted with 1 M sodium phosphate in phosphate-buffered saline solution (120 mM sodium chloride, 2.7 mM potassium chloride, 10 mM monobasic potassium phosphate; pH 7.4, at 23 °C) at a flow rate of 1.0 mL/min. Between 1 and 20 μg of protein were injected onto the column. The masses of native, monomeric HLL and soluble aggregates were calculated based on peak areas eluting at 11.2 min and between 9.1 and 11.0 min, respectively. Results were expressed as percentage of the native, monomeric protein mass present in solution prior to freeze-drying.

The amount of the protein-forming insoluble aggregates was calculated and reported as previously described.[2] To test whether protein could also be lost from solution due to adsorption to the vial or stopper during lyophilization and rehydration, the total protein content was estimated by a Bradford assay using bicinchoninic acid (BCA) (Pierce, Rockford, IL) as a reagent. For all preparations, 100% of the protein was recovered in the rehydrated formulations (data not shown), indicating that minimal protein was lost due to adsorption to surfaces.

HLL Activity Assay—HLL activity was assessed by the ability of the protein to hydrolyze tributyrin according to a slightly modified method originally described by Holmquist et al.[5,6] The amount of sodium hydroxide (Merck, Damstadt, Germany) required to neutralize hydrolyzed tributyrin (Merck, Damstadt, Germany) was monitored as a function of time employing a pH-stat titrator. The substrate solution (pH 7, at 30 °C) containing tributyrin (0.159 M) and gum arabic (0.094%, wt/vol) in water was emulsified using a blender for 20 s and subsequently stirred using a magnetic stir plate for 20–30 min. The HLL formulations were dissolved in 0.01 M glycine (Merck, Damstadt, Germany) (pH 10.6, at 23 °C) to yield a final concentration of approximately 1 mg/mL. The reaction was started by the addition of HLL solution to a stirred, thermostated (30 °C) substrate solution. The pH was maintained automatically with sodium hydroxide (0.05–0.1 M) using a Titralab 90 (Radiometer, Copenhagen, Denmark) equipped with an ABU900 autoburet (Radiometer, Copenhagen, Denmark) connected to a Titration Manager TIM900 (Radiometer, Copenhagen, Denmark). The amount of base required to maintain pH at 7.0 during 5 min of titration was recorded and used to calculate the hydrolytic activity of a given HLL formulation. Results are reported as mean values (± standard deviation) of three vials assayed in duplicate.

The enzymatic activity of HLL in solution prior to lyophilization was not determined. However, acute recovery of enzymatic activity in the lyophilized and rehydrated formulations averaged 97% ± 5% compared to a standard solution of freshly prepared HLL (1 mg/mL), and this value was therefore used as initial recovery of enzymatic activity at $t = 0$.

Karl Fisher Moisture Analysis—The relative amount of moisture was determined by preparing samples in a dry nitrogen purged glovebox and assaying for water content using a Mettler DL37 KF coulometer (Hightstown, NJ) as previously described,[2] except that slightly larger aliquots (2–5 mL) of a 1:1 (vol:vol) methanol:formamide mixture were used to dissolve the dried formulations compared to that previously used (1 mL aliquots).

X-ray Diffraction—X-ray powder diffraction was recorded with a Guinier powder diffraction camera, XDC-700 (Incentive Research & Development AB), using Cr $K\alpha_1$ (l = 2.290 Å) radiation. Small amounts of dried solid (5–10 mg) was placed in a sample disk, and the sample was subjected to radiation for approximately 30–45 min. The film was developed, and crystallization was detected as distinct band patterns characteristic for the crystallized material (sucrose).

Sodium dodecyl lauryl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), using nonreduced and reduced samples, was performed as previously described.[2] The gels showed a faint band corresponding to the molecular weight of a dimer in all stored and rehydrated formulations which had more than 20% soluble aggregates, as determined by size-exclusion chromatography (data

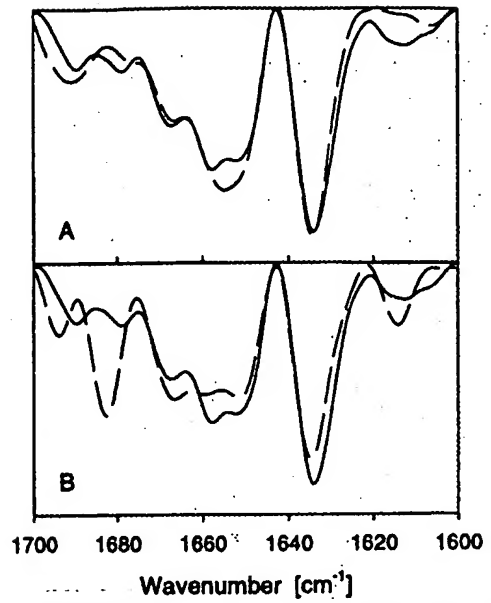

Figure 1—Area-normalized, second derivative infrared amide I spectra of native, aqueous HLL in buffer alone (10 mM phosphate buffer, pH 10, at 23 °C) (—) and of HLL precipitate (---) induced by 1 M trehalose (A) or 1% (wt/vol) Tween 20 (B).

not shown). The band was present in both nonreduced and reduced samples assayed by SDS–PAGE, indicating that the covalently bonded dimer is formed by mechanisms other than disulfide scrambling. No bands corresponding to low molecular weight fragmentation products were observed (not shown).

Infrared (IR) Spectroscopy—Infrared spectra of liquid and dried HLL formulations were recorded at 23 °C using a Nicolet Magna 550 infrared spectrometer (Madison, WI) as previously described.[2] Spectra were processed according to previously established criteria.[23]

Differential scanning calorimetric (DSC) analysis was also conducted as previously described[2] using a Perkin-Elmer DSC-7 (Norwalk, CT). Briefly, samples were heated at 10 °C/min to a final temperature of 180 °C. Selected samples were then cooled and reheated to 50–100 °C above the glass transition temperature to ensure reversibility of the glass transition. The degree of crystallinity of PEG or mannitol was calculated by dividing the enthalpy per gram of PEG or mannitol in an experimental formulation by the value obtained for the respective reference material and multiplying the result by 100.[2] Reference materials were taken from the supplier's bottle (Sigma Chemical Co., St. Louis, MO).

Results and Discussion

Effect of Additives on Secondary Structure in Solution—To determine if the presence of an additive induced secondary structural changes in HLL prior to lyophilization, protein infrared spectra in the conformationally sensitive amide I region were compared. The second derivative spectrum for the protein prepared in buffer alone is shown in Figure 1. The component bands can be assigned as follows:[24] bands at 1631, 1635, and 1689 cm^{-1} are due to β-sheet, the band at 1649 cm^{-1} is due to random elements, the band at 1657 cm^{-1} is due to α-helix, and the bands at 1668 and 1679 cm^{-1} are often assigned to turn structures. The spectra for the protein prepared in the presence of 1 M sucrose, 0.5 M mannitol, or 5% (wt/vol) dextran were essentially identical to that for the protein in buffer alone (data not shown). Thus, these additives do not alter the secondary structure of the protein in solution.

However, unexpectedly, in the presence of 1 M trehalose or 1% (wt/vol) Tween 20, the protein formed a white precipitate. Such behavior has not been reported previously in several studies of more typical globular proteins with relatively hydrophilic surfaces and may thus be attributed to the surface hydrophobicity of HLL. To gain insight into the process causing precipitation, we used infrared spectroscopy to study the protein secondary structure. The spectrum for the precipitate formed in 1 M trehalose was very similar to that for native, soluble protein, indicating that precipitation did not alter protein secondary structure (Figure 1A). Diluting the solution with buffer caused the precipitate to dissolve completely (data not shown). Thus, it appears that trehalose can "salt-out" HLL. Reversible precipitation and retention of native protein secondary structure in precipitates have previously been noted for interferon-γ salted-out with PEG,[25] and for lactate dehydrogenase, chymotrypsinogen, bovine serum albumin, and insulin salted-out with poloxamer.[26]

Salting-out of proteins by additives can be explained by the Timasheff preferential exclusion mechanism. The salting-out agents are preferentially excluded from the protein surface, which increases protein chemical potential and reduces solubility.[27] Interestingly, HLL remained soluble in 1 M sucrose but was salted-out with 1 M trehalose. Sucrose and trehalose have both been shown to be preferentially excluded to a similar degree from the surface of ribonuclease A, a protein with a more typical predominantly hydrophilic surface.[27,28]

The preferential interactions of sugars with a protein with a more hydrophobic surface such as HLL have not been studied. However, trehalose has been found to interact less favorably with poly(ethylene glycol) (PEG) than does sucrose.[29] PEG attains a more hydrophobic surface at higher temperatures due to conformational alterations.[30] Izutsu and colleagues found that trehalose depressed the cloud point of PEG (i.e., the temperature above which PEG separates as a precipitate from an aqueous solution) to a greater degree than sucrose. The lower solubility of PEG in the presence of trehalose is thought to be due to a greater repulsion between PEG and trehalose than between PEG and sucrose.[29] In turn, this difference has been attributed to differences between sucrose and trehalose stereochemistry.[30,31] That of trehalose fosters a larger volume of hydration and thus a more favorable interaction with water and a less favorable interaction with apolar molecules. Therefore, trehalose is expected to interact less favorably with hydrophobic molecules such as PEG or HLL than does sucrose.

Contrary to the trehalose-induced precipitate, the precipitate induced by Tween 20 did not dissolve upon dilution in buffer (data not shown), and the infrared spectrum of the protein precipitate was substantially altered compared to that of native HLL (Figure 1B). The presence of large infrared bands at 1614 and 1696 cm^{-1} are due to an intermolecular β-sheet,[32,33] suggesting that the precipitated protein was composed of non-native aggregates. Aggregation via the formation of non-native β-sheet structure is commonly seen for proteins under denaturing conditions such as high temperature, extremes in pH, or the presence of chaotrophs such as urea, guanidine hydrochloride, or thiocyanate.[32,33] Thus, in contrast to the "salting-out" noted in the presence of trehalose, Tween 20 alters the native conformation to a sufficient degree that the non-native molecules form intermolecular contacts and precipitate. Interaction of HLL with the Tween 20 micelle–water interface and/or direct binding of Tween 20 to the protein's surface may be responsible for perturbation of the native protein structure. For proteins with a more typical hydrophilic surface such as Factor XIII[2] and subtilisin,[34] Tween 20 does not alter the native structure of the protein.

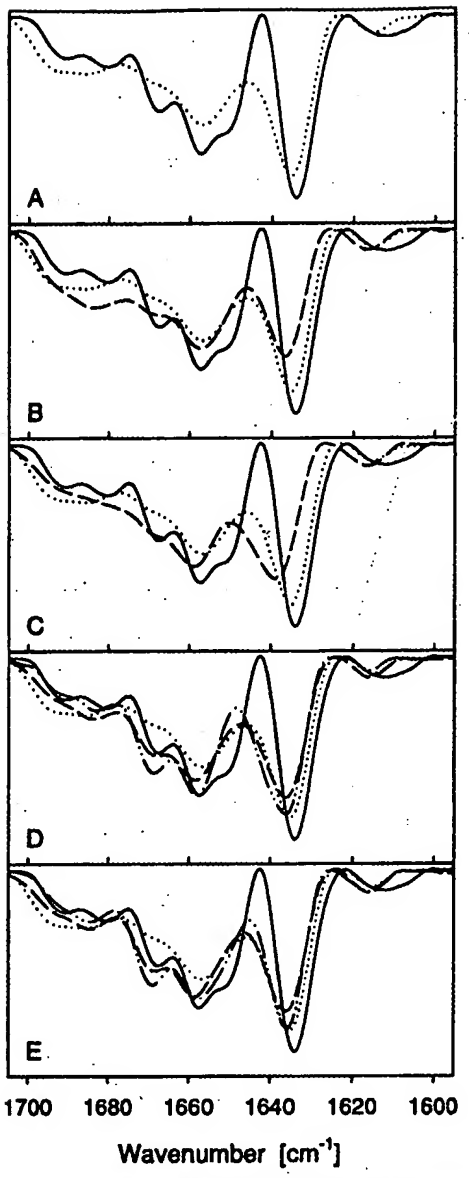

Figure 2—Area-normalized second derivative infrared amide I spectra of HLL in the native, aqueous state (—) and when lyophilized in the absence of additives (···), or in the presence of additives (---): No additives (A), 300 mM mannitol (B), 5% (wt/vol) dextran (C), 50 mM (---) or 300 mM (—···—) trehalose (D), or 50 mM (---) or 300 mM (—···—) sucrose (E).

Thus, it appears that the effects of additives on the structure and solubility of the hydrophobic protein HLL in aqueous solution are quite different from those noted with more typical soluble, globular proteins that have a more hydrophilic surface exposed to the solvent. The next question we addressed is whether different behaviors also are manifested during the lyophilization process.

Acute Stability during Freeze-Drying and Rehydration—To assess initially the effects of freeze-drying, HLL was lyophilized in the absence of additives. Figure 2A shows that the infrared spectrum of the dried protein was substantially altered relative to that of the native, aqueous protein. In general, the components bands in the amide I region for the spectrum of the dried protein appeared broader and had decreased resolution. This is typical of many lyophilized proteins.[2,35] In addition, there was a decrease in absorbance of the bands at 1635, 1649, 1657, and 1668 cm^{-1} which was compensated for by an increase in absorbance at 1645 cm^{-1} and in the region from 1684 to 1694 cm^{-1} (Figure 2A). Together, these changes indicated

Table 1—Lyophilized Formulation Characteristics

formulation	% native monomer[a]	glassy[b]	T_g,[b] °C	% moisture[a]
no additives	100 ± 0.5	yes	nd[c]	<0.5
300 mM mannitol	93 ± 1.8	no	na[d]	1.3 ± 0.5
5% (wt/vol) dextran	96 ± 1.8	yes	nd[c]	0.6 ± 0.3
50 mM trehalose	98 ± 1.6	yes	95	0.9 ± 0.7
300 mM trehalose	95 ± 1.2	yes	100	0.5 ± 0.1
50 mM sucrose	102 ± 1.2	yes	70	1.0 ± 0.5
300 mM sucrose	99 ± 0.3	yes	70	1.2 ± 0.6

[a] Values are reported as mean of three vials from each formulation (±standard deviation). Native, monomer recovery was determined by size exclusion chromatography. Moisture content was determined by Karl Fisher titration and reported as %. (g H_2O/100 g dried solid). [b] Determined by differential scanning calorimetry (see Methods). [c] nd = no glass transition detectable. Previous literature[42,43] indicates that the T_g may be above the highest storage temperature (60 °C). [d] na = not applicable, mainly crystalline material.

that HLL was unfolded in the dried solid. Upon rehydration, however, 100% native, monomeric protein was recovered (Table 1). Thus, it appeared that refolding of HLL was favored over aggregation during rehydration.[35]

To determine if the water replacement mechanism shown to account for protection of more hydrophilic proteins during lyophilization[2-4] also accounts for protection of more hydrophobic proteins, HLL was lyophilized in the presence of representative additives, which previously have been shown to (1) remain amorphous and hydrogen bond to the dried protein (trehalose or sucrose),[1] (2) fail to protect due to crystallization (mannitol),[10] or (3) remain amorphous, but fail to protect the protein because steric hindrance eliminates effective hydrogen bonding to the dried protein (dextran).[36]

The second derivative amide I infrared spectra for the protein dried with 300 mM mannitol or 5% (wt/vol) dextran were more perturbed than that for HLL lyophilized without additives (Figure 2B,C). There was a further decrease in absorbance at 1635 cm^{-1} and greater band broadening. In addition, the recovery of native, monomeric protein was slightly less with mannitol than that noted for the protein lyophilized without additives (Table 1). Dextran had little effect on the recovery of native, monomeric protein (Table 1).

Differential scanning calorimetric analysis (see Methods) documented that 85% of the mannitol mass was crystallized in the dried solid (not shown). Apparently the remaining 15% of mannitol, corresponding to a 4:1 mass ratio of mannitol:HLL, did not afford protection to the protein during lyophilization. This is consistent with results observed for more hydrophilic proteins.[2,10]

In contrast, differential scanning calorimetry showed that dextran was completely amorphous in the dried solid containing HLL (data not shown). The failure of amorphous dextran to inhibit protein unfolding may be due to steric hindrance making it unable to hydrogen bond with the dried protein. In addition, potential separation of dextran and protein into separate dextran-rich and protein-rich amorphous phases[37] could have caused greater damage to the protein during lyophilization.

Finally, the effects of sucrose and trehalose were tested. Both disaccharides formed amorphous phases (Table 1) during lyophilization and are known to have the ability to hydrogen bond to the protein during dehydration.[1,35] Infrared spectra of HLL lyophilized in the presence of either concentration (50 or 300 mM) of trehalose (Figure 2D) or sucrose (Figure 2E) were more nativelike than those of HLL lyophilized without additives (Figure 2A). The bands near 1648, 1657, and 1668 cm^{-1} were better resolved, and the absorbance in the region from 1684 to 1694 cm^{-1}

was reduced to a level more closely resembling that seen in the spectrum of the native, aqueous protein (Figure 2D,E). The spectrum of HLL lyophilized with sucrose (Figure 2E) had slightly less broadening of the 1635 cm^{-1} band compared to that seen in the spectrum of HLL lyophilized with trehalose (Figure 2D), suggesting that structural preservation by sucrose was somewhat better than that by trehalose. Thus, despite the less favorable interaction between HLL and trehalose than between HLL and sucrose in aqueous solution, similar levels of structural protection are noted during freezing-drying and rehydration. Also, slightly better band resolution was achieved in spectra of HLL lyophilized in the presence of 300 mM disaccharide compared to formulations prepared in 50 mM disaccharide (Figure 2D,E). Preliminary studies showed band resolution in the spectra of the dried protein increased when HLL was lyophilized with increasing disaccharide concentrations ranging from 10 to 300 mM (unpublished results). Finally, more than 95% of native, monomeric protein was recovered after rehydration of HLL formulations lyophilized with either disaccharide (Table 1). Taken together, these results indicate that sucrose and trehalose are far superior to mannitol and dextran at inhibiting lyophilization-induced unfolding of HLL.

Long-Term Storage Stability—It has been proposed that storage stability of a protein in the dried solid only depends on using an amorphous additive and on maintaining the formulation at a temperature below its T_g value.[38] However, in the only three published studies (with interleukin-1 receptor antagonist, Factor XIII, and interleukin-2), in which both protein structure and glass transition temperature of the dried solid have been determined,[2-4] it was found that long-term stability of a protein in the dried solid depended both on minimizing protein unfolding during the lyophilization process as well as on storage at a temperature below the formulation T_g value. If the only requirement for storage stability of HLL is that formulations must be stored in the amorphous solid at temperatures below the T_g value,[38] then formulations containing either dextran, trehalose, or sucrose should show optimal stability (Table 1). If, in addition, preservation of native structure in the dried solid is also important,[2-4] then only trehalose and sucrose should provide optimal stability (Figure 2).

However, storage stability of a dried protein may also be compromised if the sugar additive crystallizes during storage.[11,12] Sugar crystallization usually occurs at temperatures close to or above the formulation T_g value and can be minimized by increasing the mass ratio of protein to sugar.[17,18] Since the initial T_g values of the trehalose containing HLL formulations were 25–30 °C higher than those of the sucrose-containing formulations (Table 1), it would be expected that during storage at 40 and 60 °C, HLL formulations containing sucrose (especially at the higher initial sucrose concentration) would be more prone to sugar crystallization than those containing trehalose.

HLL was stored without additives to assess the storage stability of the protein alone. The additive-free formulation had a residual moisture level of less than 0.5% (g H_2O/g dried solid) (Table 1). Similar to what has been observed for other proteins,[2,17] a glass transition for the HLL formulation was not discernible by differential scanning calorimetric analysis (data not shown). The glass transition temperature of dried HLL was probably well above either storage temperature, since dried protein T_g values have been estimated to be between 100 and 200 °C.[39] Thus, if storage in the amorphous solid at temperatures below the T_g value was the only important factor accounting for long-term storage stability of the hydrophobic protein, it would

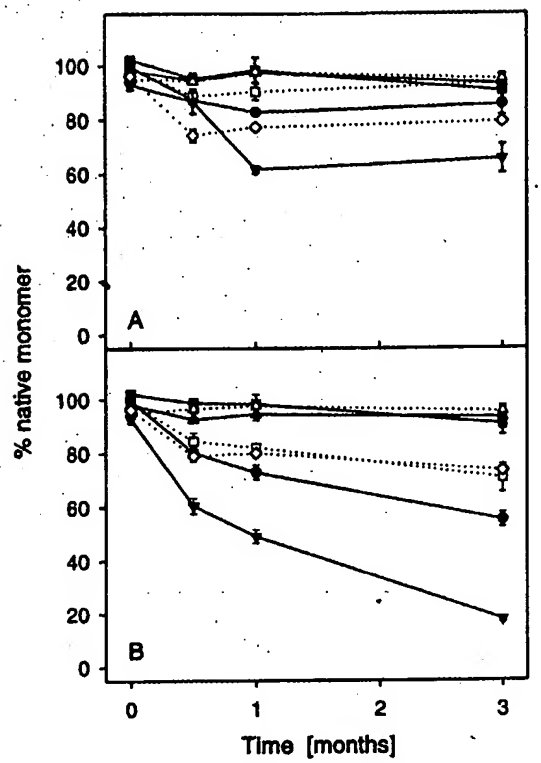

Figure 3—Recovery of native, monomeric HLL as a function of time as determined by size exclusion chromatography. The recovery was determined as the percentage of native, monomeric protein relative to that present in solution prior to lyophilization. Formulations were stored at 40 °C (A) or 60 °C (B) in the absence of additives (solid circle) or in the presence 300 mM mannitol (solid triangle, down), 5% (wt/vol) dextran (open diamond), 50 mM trehalose (solid triangle, up), 300 mM trehalose, (open triangle, up), 50 mM sucrose (solid square), or 300 mM sucrose (open square). Each data point represents the mean of 3 vials ± standard deviation.

be expected that HLL without additives would exhibit good storage stability.

Following rehydration of the formulation, which had been stored for 14 days at either 40 and 60 °C, loss of native, monomeric HLL (Figure 3) was seen. This was mainly due to formation of insoluble aggregates in the formulation stored at 40 °C (Figure 5A) and formation of approximately equal amounts of soluble and insoluble aggregates at 60 °C (Figures 4B, 5B). Larger effects were noted in the formulation stored at 60 °C than at 40 °C (Figures 3–6). There was a much larger loss of hydrolytic activity (Figure 6) compared to loss of native, monomeric protein (Figure 3) after 3 months storage. This suggested that chemical degradation in addition to physical degradation (aggregation) was taking place. Chemical degradation such as deamidation and oxidation has previously been observed in lyophilized protein formulations during storage of the dried solid.[40] Taken together, these results document that storage of HLL at temperatures below the T_g value was not sufficient to ensure optimal storage stability, similar to that noted for several other more hydrophilic proteins.[2,3,40,41]

To determine if the damage assessed after storage and rehydration was accompanied by structural alterations in the dried solid, infrared spectra of the dried formulations stored at either temperature were recorded. Figure 7A shows that the structural alterations initially induced during the lyophilization process were augmented after storage for 3 months at either 40 or 60 °C. Absorbances of the bands at 1635 and 1657 cm^{-1} were further decreased, and this was compensated for by a further increase in absorption in the 1684 to 1694 cm^{-1} region. The modest increase in absorbances at 1694 cm^{-1} might be due to an

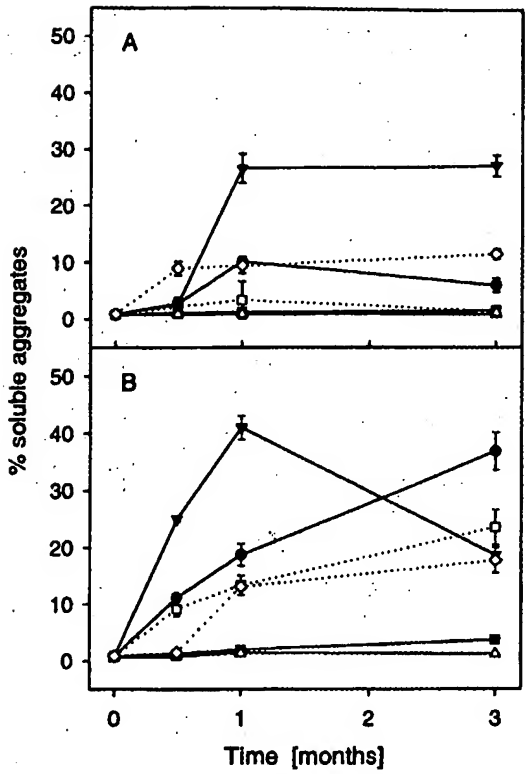

Figure 4—Formation of soluble aggregates as a function of time as determined by size exclusion chromatography. The amount of soluble aggregates formed was determined as the amount of soluble aggregates present in the stored and rehydrated formulations relative to the initial amount of native, monomeric protein present in solution prior to lyophilization. Formulations were stored at 40 °C (A) or 60 °C (B) in the absence of additives (solid circle) or in the presence 300 mM mannitol (solid triangle, down), 5% (wt/vol) dextran (open diamond), 50 mM trehalose (solid triangle, up), 300 mM trehalose, (open triangle, up), 50 mM sucrose (solid square), or 300 mM sucrose (open square). Each data point represents the mean of 3 vials ± standard deviation.

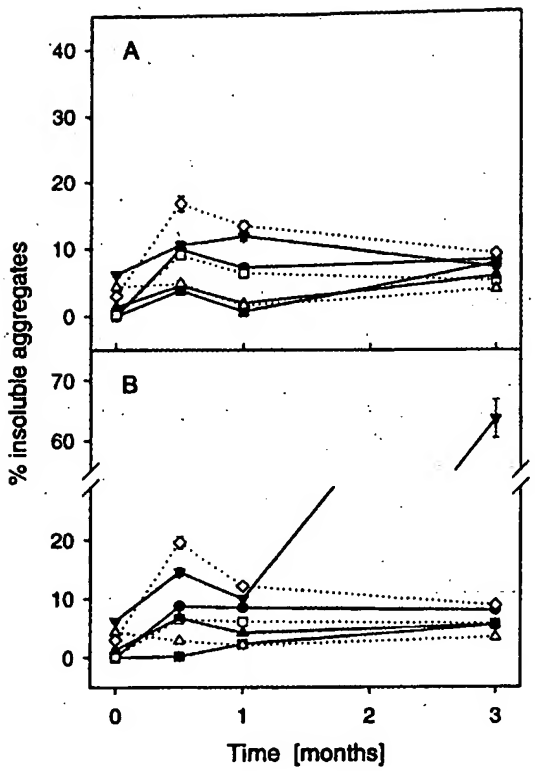

Figure 5—Formation of insoluble aggregates as a function of time. The amount of insoluble aggregates present in the stored and rehydrated formulations was calculated as the difference between the initial mass of native, monomeric protein present in solution prior to lyophilization minus the mass of recovered soluble protein (native, monomeric protein and soluble aggregates). Formulations were stored at 40 °C (A) or 60 °C (B) in the absence of additives (solid circle) or in the presence 300 mM mannitol (solid triangle, down), 5% (wt/vol) dextran (open diamond), 50 mM trehalose (solid triangle, up), 300 mM trehalose, (open triangle, up), 50 mM sucrose (solid square), or 300 mM sucrose (open square). Each data point represents the mean of three vials ± standard deviation.

intermolecular β-sheet, reflective of the formation of protein aggregates in a small fraction of the dried protein molecules.[32,33,35] However, the much greater formation of soluble aggregates noted after rehydration of the formulation stored at 60 °C compared to that measured for the formulation stored at 40 °C (Figure 4) was not reflected in the infrared spectra of the dried protein, which were very similar. Thus, it appears that aggregation during rehydration was more pronounced for the formulation stored at 60 °C.

Compared to the formulation prepared without additive, the formulation prepared with 300 mM mannitol had a greater loss of native, monomeric protein (Figure 3) and of hydrolytic activity (Figure 6) and increased formation of both soluble and insoluble aggregates during storage at either 40 or 60 °C. The one exception was the formulation stored at 60 °C which showed a decrease in the amount of soluble aggregates (Figure 4B) between 1 and 3 months storage concomitant with a large increase in the amount of insoluble aggregates (Figure 5B). Similar to that seen in the absence of additives, there was a larger decrease in recovery of hydrolytic activity (Figure 6) than that of native, monomeric protein (Figure 3), indicating that chemical degradation, in addition to physical degradation, was taking place.

Interestingly, secondary structural changes were not observed following storage for 3 months at either temperature (Figure 7B). It may have been that the amorphous mannitol in the formulation provided spatial separation between the protein molecules, thus preventing protein structural changes, resolvable by infrared spectroscopy, in the dried solid. The extensive aggregation noted after rehydration, however, indicated that mannitol was not able to inhibit aggregation during rehydration.

The poor storage stability of HLL lyophilized in the presence of mannitol is consistent with previous studies, which have shown that additives, which crystallized during lyophilization, afforded poor storage stability to several proteins.[2,10–12] The cause of the increased degradation of HLL in the mannitol formulation compared to that in the formulation without additives was not clear. There was not any increase in mannitol crystallinity during storage, based on analysis of the formulation with differential scanning calorimetry (data not shown). However, even though a glass transition was not apparent in the thermograms of the mannitol formulations, it may be that the T_g value of the amorphous solid of this formulation was lower than that for the formulation prepared without additives, resulting in reduced storage stability of the mannitol formulation.

To assess the effect of forming an amorphous solid with a T_g value above the storage temperature, HLL was stored in the presence of 5% (wt/vol) dextran. The amorphous solid formed in the presence of dextran had a moisture content of 0.6% (Table 1). No glass transition was detected by differential scanning calorimetric analysis (data not shown). Dextrans are known to form amorphous solids with T_g values increasing with the molecular weight of the dextran.[42] Since dextran with an average molecular weight of 40 kDa and a moisture content of 4% has previously been shown to exhibit a glass transition at temperatures corresponding to 90–100 °C,[43] it is assumed that the T_g value

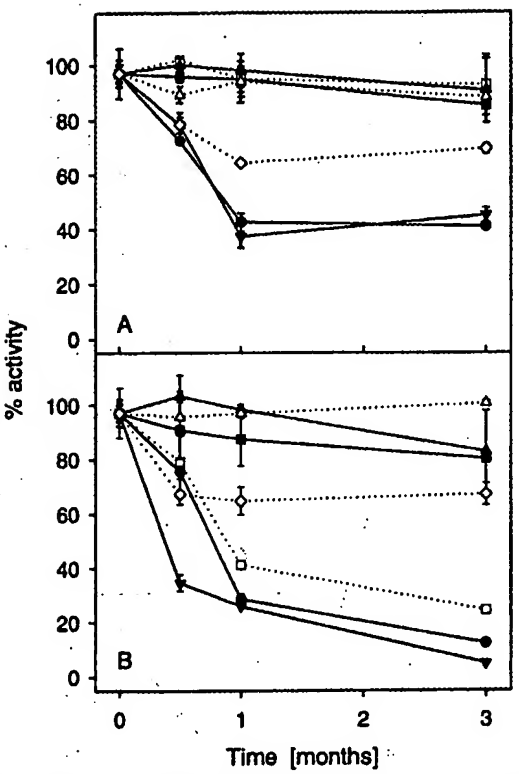

Figure 6—Recovery of hydrolytic activity as a function of time as determined by the activity assay. The recovery of hydrolytic activity was determined as the hydrolytic activity of the stored and rehydrated formulations relative to the initial hydrolytic activity of a given formulation prior to lyophilization. Formulations were stored at 40 °C (A) or 60 °C (B) in the absence of additives (solid circle) or in the presence 300 mM mannitol (solid triangle, down), 5% (wt/vol) dextran (open diamond), 50 mM trehalose (solid triangle, up), 300 mM trehalose, (open triangle, up), 50 mM sucrose (solid square), or 300 mM sucrose (open square). Each data point represents the mean of 3 vials ± standard deviation.

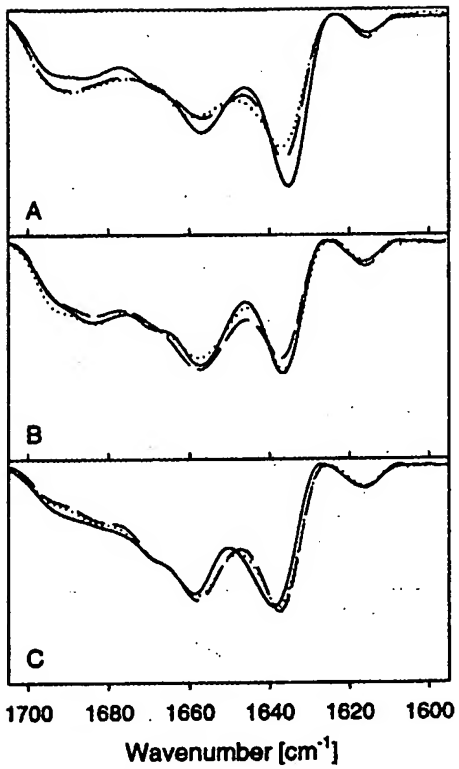

Figure 7—Effect of 3 months storage at 40 °C (···) or 60 °C (---) on the area-normalized, second derivative amide I infrared spectra of lyophilized HLL. Changes were compared to those induced during lyophilization (—). Formulations were lyophilized and stored in the absence of additives (A), or in the presence of 300 mM mannitol (B), or 5% (wt/vol) dextran (C).

of the current dextran formulation (average molecular weight of dextran = 162 kDa) was well above either storage temperature used in the current study.

Dextran did not substantially improve recovery of native, monomeric protein (Figure 3) nor decrease the formation of aggregates (Figures 4, 5) following rehydration of the formulation stored at 40 °C compared to that observed for the formulation without additives. However, a slight improvement in recovery of hydrolytic activity (Figure 6) was observed in the dextran-containing formulation stored at 40 °C. In addition, dextran slightly improved storage stability of the formulation stored at 60 °C compared to that observed in the formulation without additives (Figures 3–6). At both storage temperatures, loss of hydrolytic activity (Figure 6) following storage and rehydration was not much larger than the loss of native, monomeric protein (Figure 3), suggesting that dextran may stabilize against putative chemical degradation to a greater degree than that seen in the absence of additives.

Figure 7C shows that potential secondary structural changes arising during storage in the dried solid were not detectable with infrared spectroscopy. This is consistent with the results observed for Factor XIII[2] and interleukin-2.[4] Dextran may inhibit structural transitions in the dried solid because it forms an amorphous phase with a high T_g value, which restricts alterations in protein conformation, and also because amorphous dextran is able to spatially separate protein molecules in the dried solid.[38]

The partially improved stability of HLL in the presence of dextran is attributed to storage in an amorphous solid at temperatures substantially below the T_g value of the dextran formulation, suggesting that the same criterion, which is necessary for storage stability of hydrophilic proteins, also is important for hydrophobic proteins. However, also similar to that observed for hydrophilic proteins,[2] this mechanism alone did not appear to be sufficient for optimal storage stability of HLL.

Both concentrations of trehalose (50 mM or 300 mM) formed an amorphous cake with a low moisture content and a T_g value substantially greater than either storage temperature (Table 1), and afforded optimal stability following 3 months storage at either temperature (Figures 3–6). Minor structural alterations were induced during storage in the dried solid in the presence of either concentration of trehalose (Figure 8A,B). These alterations, however, were not reflected in the recovery of either native, monomeric (Figure 3) nor hydrolytically active molecules (Figure 6) in the stored and rehydrated formulations. This may have been because trehalose afforded an environment conducive to refolding upon rehydration.[4]

The increased storage stability relative to that noted with dextran is attributed to the greater retention of native protein structure during the initial lyophilization process (Figure 2). Thus, as has been documented for more hydrophilic proteins, for the relatively hydrophobic HLL, storage at a temperature below the T_g value of the amorphous phase as well as preservation of native structure during lyophilization are critical for storage stability.

Sucrose at either concentration (50 mM or 300 mM) formed an amorphous solid with an initial T_g value above the highest storage temperature (Table 1). In addition, sucrose was able to minimize lyophilization-induced structural alterations (Figure 2E). Figures 3–6 show that the formulation containing 50 mM sucrose stored at either temperature and also the formulation containing 300 mM sucrose stored at 40 °C were as effective at stabilizing HLL

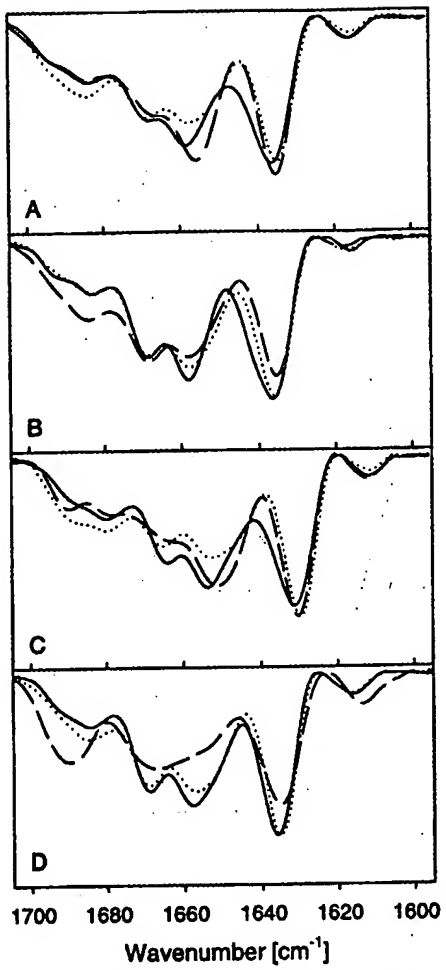

Figure 8—Effect of 3 months storage at 40 °C (···) or 60 °C (---) on the area-normalized, second derivative amide I infrared spectra of lyophilized HLL. Changes were compared to those induced during lyophilization (—). Formulations were lyophilized and stored in the presence of 50 mM trehalose (A), 300 mM trehalose (B), 50 mM sucrose(C), or 300 mM sucrose (D).

as either concentration of trehalose. However, at 60 °C, 300 mM sucrose afforded minimal stability.

Relatively small secondary structural changes were seen in the formulation containing 50 mM sucrose following storage at either temperature and in the formulation containing 300 mM sucrose stored at 40 °C (Figure 8C,D). However, the infrared spectrum of the protein stored with 300 mM sucrose at 60 °C showed substantial alterations (Figure 8D). Decreases in absorbances at 1635 and 1657 cm^{-1} were indicative of loss of native structure. In addition, increased absorbances at 1614 and 1695 cm^{-1} were due to intermolecular β-sheet formation, which suggested that aggregation was occurring in the dried solid during storage.[35] These changes were consistent with the large amount of aggregates formed upon rehydration.

Thus, at the higher storage temperature (60 °C) sucrose was able to stabilize HLL at high protein:sucrose ratio corresponding to 12% protein [g protein/g (protein+sucrose)] but not at low protein: sucrose ratio (2% protein). Differential scanning calorimetric analysis showed similar initial T_g values for the sucrose containing HLL formulations (Table 1), independent of initial protein:sucrose ratio. Similarly, Sarciaux and Hageman[17] also found that somatotropin (rbSt) did not affect the T_g value of an rbSt formulation containing sucrose at protein weight fractions below 30% (g protein/ g dried formulation). However, they did notice that increasing the concentration of rbSt at lower protein weight fractions (4–10% protein) resulted in an

Table 2—Moisture Content and Glass Transition Temperatures following 3 Months Storage

formulation	% moisture[a] (40 °C)	% moisture[a] (60 °C)	T_g,[b] (40 °C)	T_g,[b] (60 °C)
no additives	13.2 ± 0.8	11.3 ± 2.0		
300 mM mannitol	1.7 ± 0.4	1.4 ± 0.2		
5% (wt/vol) dextran	4.9 ± 0.3	6.5 ± 0.8		
50 mM trehalose	7.1 ± 0.8	4.7 ± 0.5	81	85
300 mM trehalose	2.6 ± 0.2	3.3 ± 0.4	79	87
50 mM sucrose	5.1 ± 1.3	5.9 ± 0.9	46	50
300 mM sucrose	3.1 ± 0.5	1.1 ± 0.2	52	nd[c]

[a] Moisture content of formulations stored at either 40 or 60 °C were determined by Karl Fisher titration (% [g H_2O/100 g dried solid]) and are reported as mean of three vials from each formulation (±standard deviation). [b] Glass transition temperatures (T_g) of formulations stored at either 40 or 60 °C were determined by differential scanning calorimetry (see Methods) and are reported as an average temperature of two vials from each formulation. [c] nd = no glass transition detectable.

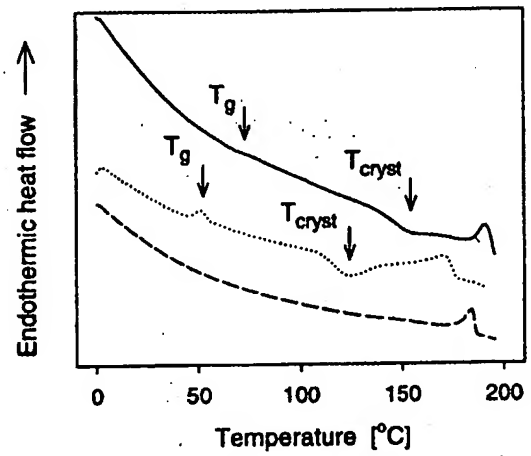

Figure 9—Differential scanning calorimetric thermograms of HLL formulations lyophilized with 300 mM sucrose: Immediately after lyophilization (—), or after 3 months storage at 40 °C (···) or 60 °C (---). Formulations containing amorphous sucrose prior to thermal scans (top and middle thermograms) exhibited a second-order transition corresponding to a glass transition (T_g) and an exothermic peak (T_{cryst}) corresponding to sucrose crystallization during heating in the calorimeter. The formulation containing crystalline sucrose prior to thermal scanning did not exhibit either of these thermal events (lower thermogram). The endothermic peak at the glass transition of the formulation stored at 40 °C (···) is due to enthalpic relaxation.

increase in the crystallization temperature of sucrose. Thus, for the current study, although no measurable effect were resolved based on the initial T_g values (Table 1), the differences in storage stability of the two sucrose formulations may be due to inhibition of sucrose crystallization in the formulation containing the greatest weight fraction of HLL.

Crystallization of a sugar commonly requires storage at or above the formulation T_g value.[13] Although the initial T_g values of the dried disaccharide formulations were all above the highest storage temperature (Table 1), during storage the formulations showed an increase in moisture content and a concomitant decrease in the T_g values (Table 2), presumably due to moisture transfer from the stopper.[44]

Differential scanning calorimetric analysis indicated that the decrease in T_g values for both of the trehalose formulations and for the formulation containing low concentrations of sucrose was not accompanied by crystallization of sugar (not shown). In contrast, after 3 months of storage at 60 °C, the 300 mM sucrose formulation did not show a glass transition nor was a crystallization exotherm observed in the thermogram (Figure 9). For comparison, these features were clearly apparent in thermograms for the corresponding formulation immediately after lyophilization or after

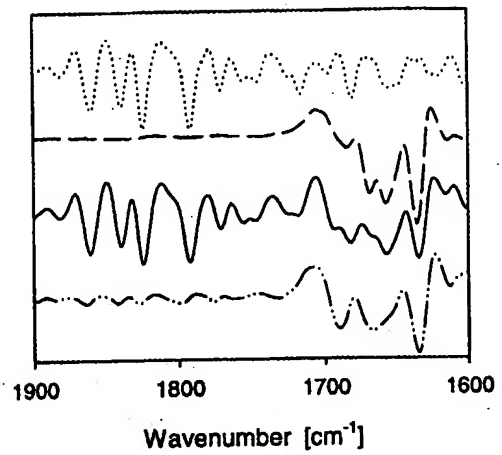

Figure 10—Area-normalized second derivative infrared spectra (1900 cm^{-1} to 1600 cm^{-1}) of crystalline sucrose obtained from the supplier's bottle (···) and of HLL, lyophilized in the presence of 300 mM sucrose, immediately after lyophilization (—), and after 3 months storage at 60 °C (—). Subtraction of the crystalline sucrose spectrum from the spectrum of HLL lyophilized with 300 mM sucrose, stored at 60 °C for 3 months, resulted in a nearly flat baseline from 1900 cm^{-1} – 1700 cm^{-1} (–··–), ensuring that absorbance in the amide I region (1700 cm^{-1} – 1600 cm^{-1}) was due only to protein.

storage at 40 °C (Figure 9). Thus, for the 300 mM sucrose formulation, the amorphous saccharide had crystallized during storage at 60 °C. This conclusion was confirmed with X-ray powder diffraction, which showed that sucrose crystallization was initiated in the formulation containing 300 mM sucrose following 1 month storage at 60 °C and increased further after 3 months of storage (data not shown). There was not any crystalline sucrose detectable by X-ray powder diffraction in the 300 mM sucrose formulation immediately after lyophilization. And there was no indication of crystalline sucrose in the formulation containing 50 mM sucrose, even after 3 months of storage at 60 °C (data not shown).

Further evidence for sucrose crystallization was obtained by infrared spectroscopy. We have previously shown that sucrose crystallization can be detected by analyzing the second derivative IR spectra in the 1900–1700 cm^{-1} region.[2] Figure 10 shows the second derivative IR spectra of crystalline sucrose obtained from the supplier's bottle and of noncrystalline and crystalline sucrose containing HLL formulations. Both the spectrum of pure crystalline sucrose and that of the HLL formulation containing crystalline sucrose (300 mM sucrose formulation stored for 3 months at 60 °C) had strongly absorbing bands at 1860, 1839, 1824, and 1792 cm^{-1}. In contrast to this, spectra of HLL containing only amorphous sucrose (e.g., immediately after lyophilization) showed a straight baseline in the 1900–1700 cm^{-1} region (Figure 10). Since crystalline sucrose also absorbed slightly in the amide I region (1700–1600 cm^{-1}), it was necessary to subtract the spectrum of crystalline sucrose from that of the HLL formulation containing crystalline sucrose. The subtraction result showed a relatively flat baseline between 1900 and 1700 cm^{-1}, indicating that absorption of the resultant spectrum in the amide I region was due to protein only (Figure 10).

The increased damage to HLL associated with crystallization of sucrose most likely is due to the fact that crystalline sucrose is anhydrous.[45] Thus, as sucrose crystallizes the relative mass of water remaining in the amorphous phase will increase. As a result, the T_g value of the remaining amorphous material will decrease, resulting in compromised protein stability. Similar results were noted with formulations of Factor XIII in which sucrose crystallized.[2]

Finally, the formulation containing 50 mM sucrose stored at 60 °C for 3 months had a final T_g value below the storage temperature (Table 2), yet no sucrose crystallization was observed. This may have been due to several factors. First, when stored at a temperature near the T_g value, the formulation may collapse, but not crystallize.[42] Second, as explained above, increased weight fractions of protein in a dried solid may increase the crystallization temperature without affecting the T_g value.[17,18] Thus, it may have been that the crystallization temperature of sucrose in the current HLL formulation remained above the highest storage temperature (60 °C).

Conclusion

The precipitation of HLL—by salting-out action of trehalose and denaturing action of Tween—documents that the effect of excipients on this relatively hydrophobic protein are much different than those seen with proteins with more typical predominantly hydrophilic surfaces. Despite this difference, the same mechanisms accounting for both acute and long-term storage stability of hydrophilic proteins also accounted for stability of HLL. First, optimal acute stability during lyophilization of HLL required the formation of an amorphous phase containing protein and additive. In addition, the additive should possess the ability to hydrogen bond and thus act as a water substitute during dehydration. However, since the protein favored refolding over aggregation upon rehydration, damage to HLL during lyophilization was best resolved in the dried solid by infrared spectroscopy. Next, formation of an amorphous phase with a T_g value above storage temperature and preservation of native structure during lyophilization were both necessary criteria for storage stability. However, as was seen for hydrophilic proteins,[10–12] crystallization of an amorphous saccharide during storage resulted in poor storage stability. Crystallization of sucrose could be prevented by increasing the protein:sucrose mass ratio, which presumably resulted in an increase in the crystallization temperature. Finally, the superior stabilization afforded by trehalose compared to that afforded by sucrose was attributed to the higher T_g value of the trehalose-containing formulation.

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